PRODUCTION OF MONOCLONAL ANTIBODIES TO SEROGROUP 6 OF LEGIONELLA PNEUMOPHILA (STRAIN CHICAGO 2)

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COMNUAN NOKKAEW

DEPARTMENT OF BIOLOGY

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MASTER OF SCIENCE

OF

COMNUAN NOKKAEW

APPROVED:

Major Professor Judith Lee Lenk

Thesis Committee Member James H. Penn

Thesis Committee Member Roger M. McKinney

Department Chairman Rosalyn M. Patterson

Dean, School of Arts and Sciences _____

ABSTRACT

BIOLOGY

NOKKAEW, COMNUAN B.S., KASETSART UNIVERSITY, 1975

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Production of Monoclonal Antibodies to Serogroup 6 of Legionella pneumophila (Strain Chicago 2)

Advisors: Dr. Judith Rae Lumb and Dr. Roger M. McKinney

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Legionellosis is an acute respiratory disease which occurs throughout the United States and other areas of the world. The etiologic agent of this disease is a gram-negative, fastidious bacterium that belongs to the genus Legionella. The most common species, L. pneumophila, has been divided into 7 serogroups. Strains of serogroups 3 and 6 show common antigens as indicated by cross-reactivity which has been obtained with rabbit antisera. The objective of this research is to produce monoclonal antibody against cell surface antigens of L. pneumophila serogroup 6 (Chicago 2 strain), to determine whether there is antigenic heterogeneity among strains of L. pneumophila serogroup 6, and to determine whether monoclonal antibodies to serogroup 6 might be useful as diagnostic reagents for detecting legionellosis.

Hybridoma cell lines producing monoclonal antibodies for the determinants of L. pneumophila serogroup 6 were derived from the fusion of mouse myeloma cells and spleen cells from mice immunized with

L. pneumophila Chicago 2 strain. Test sera of mice immunized with Chicago 2 strain were negative against the Bloomington 2 reference strain of L. pneumophila serogroup 3. After fusion, the suspension was plated in 10 plates. Screening of Chicago 2 antibody-producing clones by indirect fluorescent antibody (IFA) was performed throughout the project. The clones producing the highest fluorescence in each plate were recloned twice to insure monoclonality. Ten clones producing monoclonal antibodies against Chicago 2, the representative strain of L. pneumophila serogroup 6, were isolated. These were tested by IFA against 30 strains of L. pneumophila serogroup 6 and 6 strains of L. pneumophila serogroup 3. All ten of the monoclonal antibodies gave positive results to 30 strains of serogroup 6 but not to 6 strains of serogroup 3.

It is concluded that monoclonal antibody is superior to the conventional antisera because of the specificity demonstrated. The reaction of monoclonal antibodies with 30 strains of L. pneumophila serogroup 6 demonstrated that 3 strains showed antigenic variation. These results also indicate the potential of monoclonal antibodies as diagnostic reagents for identification of L. pneumophila.

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CHAPTER I

INTRODUCTION

Legionnaires' disease (LD) is an infectious disease caused by any member of the pathogenic organisms that belong to the genus Legionella. The pneumonic form of LD is an acute severe respiratory illness (Thacker, et al., 1978). Pneumonic LD is not readily distinguishable from other bacterial pneumonias on the basis of clinical symptoms. About 25,000 cases of LD occur in the U. S. per year. Of these cases of known outcome, between 12-20% of cases result in death (Foy, et al., 1979). More than 500 sporadic cases of LD have been reported to the Centers for Disease Control (CDC) from at least 43 states and the District of Columbia. Nine species of organism have been reported: L. pneumophila (Brenner, et al., 1979), L. bozemanii (Brenner, et al., 1980) L. dumoffii (Brenner, et al., 1980), L. micdadei (Hebert, et. al., 1980b), L. gormanii (Morris, et al., 1980), L. longbeachae (McKinney, et al., 1981), L. jordanis, (Cherry, et al., 1982) L. wadsworthii (Edelstein, et al., 1982), and L. oakridgensis (Orrison, et al., 1983).

L. pneumophila has been divided into 7 serogroups (McKinney, et al., 1979; Bibb, et al., 1983). Serogroups 1-6 were defined on the basis of fluorescent antibody staining (McKinney, et al., 1979). The strain, Chicago 2, is the representative strain of serogroup 6 and appears to be widely distributed geographically (McKinney, et al., 1979). Chicago 8 is the representative strain of serogroup 7 (Bibb, et al., 1983). There may be additional undocumented serologic variants of L. pneumophila around the

world which are all potentially highly dangerous to human health (McKinney, R.M., Personal communication). Epidemiologically, Legionella organisms have been isolated from water, from cooling towers, evaporative condensers in air conditioning systems and from streams and natural water reservoirs. Outbreaks of this disease usually occur in hospitals and hotels (Blackmon, et al., 1979). The clinical signs of this disease are not specific and differential diagnosis is complicated. A sensitive and specific method for detection of this organism would be of great advantage in diagnosis and ultimately in elimination of the disease.

Direct fluorescent antibody (DFA) testing has proved to be a sensitive and specific method for detecting Legionella (Cherry and McKinney, 1979). However, serogroups 3 and 6 show common antigens as indicated by cross reactivity when tested in the DFA test using conjugated rabbit antisera. Isolates from serogroups 1, 2, 4, and 5 do not react with conjugates prepared against isolates of serogroups 3 and 6. Although cross-reactivities between serogroups 3 and 6 exist, absorption with a strain of one serogroup can eliminate all cross-reactivities to that serogroup. This, however, reduces the working concentration of these conjugates. Frequently, sera from patients with LD do not contain detectable antibodies against the identified infecting strain of the Legionnaires' disease bacterium (LDB), whereas, sera from some other patients infected with dissimilar pathogens may contain antibodies that react with LDB, either because of similar antigens or perhaps because of nonspecific stimulation of the reticuloendothelial lymphocytic system (Wilkinson, et al., 1979). This is a second difficulty in diagnosis of LDB by immunological techniques. Tests such

as radioimmunoassay (RIA)(Kohler, et al., 1981) and enzyme-linked immunosorbent assays (ELISA) (Tilton, 1979; Sathapatayavongs, et al., 1982) show much promise as rapid diagnostic tests for soluble antigens of L. pneumophila in urine. The success of these tests is highly dependent on the availability of potent and specific antibodies directed against the appropriate antigens of the Legionella organisms. For these reasons, the FA test, ELISA test and RIA assays should be used in conjunction with monoclonal antibodies which can be obtained with hybridoma technology.

Antibodies that can be used throughout the world as standardized diagnostic reagents are highly desirable. This has been made possible by using hybridomas that secrete monoclonal antibody of desired specificity, which can be obtained in unlimited amounts. Lymphocytes are not maintained in cultures for very long but a technique has been developed (Kohler and Milstein, 1975, 1976), whereby a single antibody-producing cell can be propagated indefinitely in culture by hybridization with a tumor cell. Since all of the cells of one clone are derived from a single lymphocyte, "monoclonal" antibodies of high purity are produced. A monoclonal antibody can detect specifically a particular epitope of the set of antigenic determinants thereby eliminating some of the problems of cross-reactivity encountered using conventional antisera. Monoclonal antibody produced by hybridomas derived from the fusion of mouse myeloma and spleen cells of mice that have been immunized with previously defined antigen might react with higher specificity to that antigen than would conventional antisera.

The purpose of this research was to use hybridoma techniques to produce monoclonal antibody against cell surface antigens of L. pneumophila serogroup 6 (Chicago 2 strain), to determine whether there is antigenic heterogeniety among strains of L. pneumophila serogroup 6, and to determine whether monoclonal antibodies to serogroup 6 might be useful as a diagnostic reagent for detecting legionellosis.

CHAPTER II

REVIEW OF LITERATURE

Diagnosis of Legionellosis

The term "Legionnaires' disease" (LD) is used to refer to the illness caused by the gram-negative bacteria of the genus Legionella. The outbreak in Philadelphia in 1976 brought LD worldwide attention and led to the isolation of the Legionnaires' disease bacteria (LDB) at the Centers for Disease Control (CDC). LDB is a gram-negative, fastidious organism. It does not grow on most bacteriological media or grows very slowly (McDade, et al., 1977), but it can be cultivated on charcoal yeast extract (CYE) agar (Feeley, et al., 1978).

Isolation of LDB. Originally LDB was isolated in guinea pigs and propagated in embryonated hen eggs (McDade, et al., 1979). LDB can be confirmed by growth on CYE agar, by failure to grow on trypticase soy agar or blood agar base media, by cellular fatty acid composition (Moss, et al., 1974), and by studies of DNA relatedness to the type strains of the Legionella species.

Morphology of LDB. The morphology of the LDB varies widely under different growth conditions. The cellular morphology changes progressively when LDB is grown in broth. Large masses of filaments or chains of bacilli in the logarithmic phase break into shorter filaments and ultimately form single and double cigar-shaped cells. With prolonged incubation or limited substrate, cells become coccal shape. No spores or microcysts are formed but swollen cell terminals were observed (Pine, et al., 1979).

Nomenclature and Taxonomy of LDB. LDB was named Legionella pneumophila sp. nov., the type species of genus Legionella (Brenner, et al., 1979). The genome size of LDB DNA was estimated at 2.5×10^9 daltons by reassociation kinetics; a GC content of 39% for LDB DNA was established by optical thermal denaturation and buoyant density ultracentrifugation measurements (Brenner, et al., 1978). New organisms, WIGA, Mi-15, LS-13, NY-23, Tex-KL, TATLOCK, HEBA and Pittsburgh pneumonia agent, were isolated and resemble L. pneumophila phenotypically (Cordes, et al., 1979; Thomason, et al., 1979; Hebert, et al., 1980a and Hebert, et al., 1980b). Brenner, et al. (1980), have proposed these organisms be initially described as Legionella-like organisms (LLOs) until they are assigned a permanent name. L. bozemanii has been proposed for WIGA and Mi-15 (Brenner, et al, 1980), L. dumoffii for NY-23 and Tex-KL (Brenner, et al., 1980), and L. micdadei for TATLOCK, HEBA, and Pittsburgh pneumonia agent (Hebert, et al., 1980c).

The LDB is clearly prokaryotic (Murray, 1974) in that it lacks eukaryotic features such as mitochondria, nuclear membranes, endoplasmic reticulum, and mitotic division. Prominent features include electron-lucent, filamentous nucleoides interspread among areas of well-defined ribosomes; enclosure by a double envelope, each portion of which consists of a triple layered "unit membrane" (Glavert, 1962) and division by a pinching, non-septate process. This pinching type of division and double envelope enclosure are characteristics of gram-negative bacteria (Glavert, 1962; Steed and Murray, 1966). Electron microscopy shows that LDB is a blunt or tapering rod. Because the ultrastructure of the LDB is not unique, it may be impossible to differentiate this organism from

other small gram-negative bacilli by fine structure alone. The width is approximately 0.5-0.7 μm. The length varies from 2-20 μm or more and the organisms contain flagella as well as flagellum-like appendages. The cells appear red when counterstained with carbol fuchsin and light pink when counterstained with safranin.

Epidemiology. Investigations of several outbreaks indicated that the etiological agent was transmitted in air. Among the environmental sources implicated were water from air conditioning cooling towers or evaporative condensers (Glick, et al., 1978) and soils disrupted by construction work and presumably transported as dust by air currents (Thacker, et al., 1978).

In 1965, an outbreak of an epidemic similar to LD occurred a St. Elizabeth's Hospital where 81 patients became ill, and 14 died. In 1968, 144 employees and visitors who entered a health department building in Pontiac, Michigan became ill. In 1973, an estimated eight Scottish vacationers contracted a disease similar to LD while vacationing in Spain; three died. In 1974, people who attended a convention at Philadelphia developed pneumonia and two died. Retrospectively, serologic evidence was used to show that all of these outbreaks were epidemics of LD. In 1976, the American Legion Convention was held in the Philadelphia's Bellevue Stratford Hotel; 182 became ill and 29 died. The outbreak in Philadelphia in 1976 brought LD worldwide attention and led to the isolation of the LDB at the CDC (McDade, et al., 1977).

Clinical Features. The most common clinical sign in LD is pneumonia. The onset is usually 2 to 10 days after exposure to LDB, with malaise, myalgia and mild headache. A non-productive cough is common,

but sputum production is sometimes associated with the disease. Within less than a day, the patient may experience rapidly rising fever and onset of chills. Fever to 39-41 C (102-105 F), and relative bradycardia. There is no physical finding specific to this disease. Associated manifestations may include confusion, chest pain, abdominal pain, impaired renal function, and diarrhea.

Direct Fluorescence Assay (DFA). Autopsy and biopsy tissue can be screened for LDB more quickly by the DFA test than by pathological examination. In addition, the DFA test has a dimension of serological specificity not possessed by the Dieterle silver impregnation stain or other histological techniques used to demonstrate the organisms in tissue. DFA staining has proved to be a sensitive and specific method for detecting LDB in both clinical specimens and environmental samples (Cherry and McKinney, 1979).

Indirect Fluorescence Assay (IFA). The difficulty in isolation of Legionella species from patients has caused heavy dependence on immunological techniques for diagnosis of legionellosis. The IFA test was first used by McDade, et al., (1977) to provide serological evidence that an organism which has been isolated from patients with LD was actually the causative agent of that disease. IFA tests for serum antibody are continuing to be adapted (Wilkinson, et al., 1979).

Hybridoma Technology

A new era of immunology evolved with a report describing "Continuous Culture of Fused Cells Secreting Antibody of Predefined Specificity," by Kohler and Milstein, (1975). This classic report led to the application

of monoclonal antibodies in many areas of biology and medicine. In 1978, a Lymphocyte Hybridomas meeting was held in Bethesda, Maryland. Many investigators reported the fusion of myeloma to immunized spleen cell from animals and production of monoclonal antibodies reacting with a broad variety of antigenic determinants. A succession of studies led to this important development. The most important include the adaptation of a myeloma line and the use of an hypoxanthine guanine phosphoribosyl transferase negative ($HGPRT^-$) parent myeloma to allow for selection of somatic hybrid by Littlefield (1964).

Adaptation of Myeloma Line. Plasmacytomas can be introduced into culture, cloned, and maintained as continuously growing relatively homogenous culture lines. The P3K continuous line was established by Horibata and Harris (1970). The MPC-11 continuous line was established by Margulies et al. (1976a) soon after P3K. P3 and MPC-11 were studied widely both in terms of immunoglobulin synthesis and stability of the cell line. It was found that these cell lines were unstable and had a high rate of loss of H chain synthesis and stability of the cell line. Non-secreting mutants were also found to occur in high frequency.

Use of $HGPRT^-$ Mutant for Selection of Hybrid. Littlefield (1964) was the first investigator who demonstrated how to select somatic cell hybrids. There are two sublines from mice known as the L-Lines: one resistant to 8-azaguanine, the other to 5-bromodeoxy uridine (BUdR) (30 μg/ml). These two drug resistant lines are correlated, respectively, with deficiencies for the enzymes HGPRT and thymidine kinase (TK) which are required for phosphorylation of base analogs. Mammalian cells have two pathways of synthesis of nucleotides: the de novo pathway, whereby

nucleotides are synthesized from sugars and amino acids, and the "scavenger" pathway, which utilizes the preformed nucleotides, hypoxanthine, and thymidine. The de novo pathway can be blocked by aminopterin. The operation of the scavenger pathway depends on the simultaneous presence of TK and HGPRT. Therefore, the drug resistant and $HGPRT^-$ parent cells are unable to grow in a medium containing hypoxanthine, aminopterin, and thymidine (HAT). On the contrary, the hybrids which contain the genes of both parents and, therefore, produce both TK and HGPRT, grow unhampered in HAT.

Production of mutant myeloma cells lacking of HGPRT is relatively easy because the enzyme is coded for by a gene on the x chromosome. Mammalian cells possess only one active x-chromosome (lysonisation). Thus, only a single mutation is needed to result in total loss of the enzyme. Thioguanine or 8-azaguanine have been used to select for $HGPRT^-$ mutants of myeloma cells. Either drug will be toxic only to cells that have an intact scavenger pathway. $HGPRT^-$ mutants will thus resist the effect of the drug and survive using the de novo pathway.

When two antibody-producing cells are fused, the products of both parental lines are expressed. Each immunoglobulin chain results from the integrated expression of one of several V and C genes coding respectively for its variable and constant sections (Cotton and Milstein, 1973). In view of the production of heavy chains from myeloma parent lines, it was desirable to develop a non-secreting myeloma line. Sp2/0-Ag14, was isolated as a re-clone of Sp2/HL-Ag, itself derived in several steps from Sp2/HLGK, a hybrid between a BALB/c spleen cell contributing a IgG2b(H) and K(L) chain with anti-sheep blood cell activity and a myeloma cell

line X63-Ag8 IgG_1(H) and K(L) (Kohler and Milstein, 1975). Sp2/0-Ag 14 is resistant to 20 μg/ml of 8-azaguanine, dies in HAT supplement medium and synthesizes no Ig chains (Kearney, et al., 1979). It has about 73 chromosomes, which is only eight more than the chromosome number of X63-Ag8, a cell line commonly used to generate hybridomas (Schulman, et al., 1978).

Fusing Agents. Sendai virus was first used for promoting fusion by Harris and Watkins (1965). Later on, many investigators attempted to fuse mouse myeloma with mouse myeloma by using Sendai virus for promoting fusion. These were for the most part unsuccessful because few mouse myelomas contain receptors for Sendai virus. Cotton and Milstein (1973) were successful in fusing mouse and rat myeloma by using Sendai virus. These hybrids continued to produce mouse heavy and light chains and rat light chain.

Kohler and Milstein (1975) and Margulies, et al., (1976b) succeeded in fusing mouse myeloma with mouse myeloma by using Sendai virus as the fusing agent. True hybrids had been formed. These experiments led to the new era of immunology in studying of monoclonal antibody. The resulting hybrids had three major characteristics:

(1) Hybrids can be formed between myeloma parent cells;

(2) The expression of immunoglobulin chains produced by both parental cells lines occurred; and

(3) The total number of chromosomes in the hybrids is less than the sum of the parental complements, indicating chromosome loss.

The hybridization techniques continued to improve. Davidson and Gerald (1976) improved techniques for induction of mammalian cell hybridization by polyethylene glycol (PEG). They reported that the concentration and time of exposure to PEG were important and that fusion frequencies could be increased appreciably under appropriate condition. Norwood, et al., (1976), reported that the fusion efficiency was high in the presence of 5% DMSO. Fazekas and Scheidigger (1980) also reported that lower molecular weight PEG proved less efficient and some of them were obviously toxic. Temperature and pH of the medium also influenced the fusion efficiency. Thirty and fifty percent PEG concentrations were recommended by many investigators.

CHAPTER III

MATERIALS AND METHODS

Figure 1 shows a flow chart of the basic method used to fuse immunized mouse spleen cells with 8-azaguanine-resistant myeloma cells (Sp2/o-Ag14). Chicago 6, the representative strain of organisms in serogroup 6, was used as an immunogen. Polyethylene glycol (M.W. 1000) is used as the fusing agent and hypoxanthine, aminopterin, and thymidine medium is used for selecting stable, monoclonal antibody producing hybrid cell lines.

Antigen Preparation

Chicago 2 strain of Legionella pneumophila (serogroup 6) was obtained from Dr. Roger McKinney, Supervisor of the Immunodiagnostic Methods Laboratory, Biotechnology Branch, Centers for Disease Control, Atlanta, Gerogia. These organisms were subcultured in Buffered Charcoal Yeast Extract (BCYE). The innoculation slants were incubated at 37 C for about 48 hours. The organisms were inoculated into tryptic soy agar and incubated at 37 C for about 48 hours in order to check for contamination by other organisms. The antigen identity was confirmed by Direct Fluorescent Antibody (DFA) test. One percent of formalin in phosphate buffered saline (PBS, Appendix F) was used to harvest and inactivate this antigen. It was centrifuged at 2,000 rpm for 30 minutes and the pellet was resuspended in 0.5% formalin PBS (Appendix F). The turbidity of the cell suspension was compared with McFarland Standard #3 (McFarland Nephelometer Density Standards; approximately 9×10^8 cells/ml). For

Fig. 1. Flow chart indicating steps in the production of hybrid cell lines secreting monoclonal antibody.


SEED Sp2/O-Ag14 CELLS
HARVEST Sp 2/O-Ag14 CELLS
IMMUNIZE MICE (CHICAGO 2 ANTIGEN)
PREPARE A SINGLE CELL SUSPENSION OF SPLEEN CELLS
FUSE SPLEEN AND Sp2/O-Ag14 CELLS WITH PEG 1000
PLATE FUSION SUSPENSION (96-WELL PLATE)
SCREEN (IFA, CHICAGO 2)
RECLONE (96-WELL PLATE)
SCREEN (IFA, CHICAGO 2)
EXPAND POSITIVE CLONES (24 WELL PLATE)
EXPAND POSITIVE CLONES (T-25 FLASK)
EXPAND POSITIVE CLONES (T-75 FLASK)
CHARACTERIZATION (IFA, STRAINS of SEROGROUPS 3 and 6)

injection, a portion of the antigen suspension was mixed with an equal volume of Freund's complete adjuvant (Difco Laboratories, Lot #0638-60) in Mulsi Churn Luer Lock syringe (Mulsi Jet Inc., Elmhurst, III.).

Production of Antisera in Mice

The emulsified immunogen was transferred into a tuberculin plastic syringe before injection into mice. The remainder of emulsified immunogen was kept for one month in order to check for separation of emulsifying agent and the antigen. Twelve-to-fifteen-week old female BALB/c mice were used. Each mouse was injected intraperitoneally with approximately 9×10^7 cells. Each mouse was marked for identification. A booster, with 1.8×10^8 cells of antigen suspended in 0.5% formalin PBS (Appendix F), was given intravenously 30 days after the first immunization and four days before undertaking fusion. The titer of antibody was checked before the booster injection and before fusion in order to determine which mice gave rise to the highest titer. Once the titer was determined, the three mice with the highest titer were killed and their spleens removed to obtain antibody producing B-lymphocytes.

Preparation of Peritoneal Macrophages

BALB/c mice were killed, sterilized with 70% alcohol and the abdominal skin was removed, leaving the peritoneum intact. The peritoneum was then sterilized with 70% alcohol. Five ml of Hanks Balanced Salt Solution (HBSS) without Ca++ or Mg++ were injected into the peritoneal cavity with a 26 gauge, 3/8 inch needle. After gently massaging the abdomen or shaking and pulling the mouse's legs, the fluid was withdrawn.

The peritoneal macrophage cells were collected in a 50 ml centrifuge tube (Corning). The cells were washed with 40 ml Dulbecco's Modified Eagle Medium (DMEM) containing antibiotics by centrifugation at 800 rpm for 10 minutes at room temperature. The cell pellet was resuspended in 10 ml of DMEM with hypoxanthine-aminopterin-thymidine (HAT) (Appendix C). The suspension was counted and adjusted to contain 1.5×10^5 cells/ml (equal to 1.5×10^4 cells/well). Cells were plated by dropping 2 drops with a 10 ml pipette (approximately 0.1 ml) of peritoneal macrophage cells into each well of 96-well tissue culture clusters with flat bottom wells (Costar, 96-well plate). Preparation of peritoneal macrophage cells was done 24 hours before fusion and the plates were checked for contamination before preparation of spleen cells.

Preparation of Spleen Cells

Animals were killed by cervical dislocation. Seventy percent ethanol was used to sterilize the animals. Each animal was placed facing up in a sterile petri dish. The skin was pinched at the stomach area and cut with scissors. The skin was pulled aside on both sides of the slit and split horizontally. The peritoneum area was disinfected with 70% alcohol and then the peritoneum which covered the spleen was removed. The spleens were removed, minced with sterile scissors, and pressed through the screen with a sterile spatula. Ten ml cold DMEM with antibiotics without Fetal Bovine Serum (FBS) (Appendix D) was used to suspend the cells. The cell suspension was drawn sequentially through 19, 21, 23, and 25 gauge needles in order to separate the spleen cells. The cell suspension was redrawn with the 25 gauge needle and discharged into a 50 ml

centrifuge tube (Corning 50 ml/centrifuge tube with screw cap, polypropylene 25330). The volume was brought to 50 ml with cold DMEM containing antibiotics. The cells were pelleted by centrifugation at 800 rpm for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 30 ml DMEM and recentrifuged. Again, the cell pellet was resuspended in 5 ml of DMEM. Cells were counted and adjusted to 2×10^7 cells/ml in 5 ml of solution. The prepared cells were incubated in a 37 C water bath until fusion was accomplished.

Preparation of Myeloma Cells

Mouse myeloma cells (Sp2/0-Ag14) for use in hybridoma production were obtained from Mr. David E. Wells, of the Centers for Disease Control, Atlanta, Georgia. Three vials of myeloma cells were thawed out of liquid nitrogen and dipped quickly in a 37 C water bath. As soon as liquified, the cells were taken out with a sterile pasteur pipette and they were transferred into a 50 ml centrifuge tube containing cold growth medium. The cells were pelleted and washed again with cold medium. Cell monolayers were grown in Tissue Culture Flasks with 25 cm^2 growth area (Falcon, lot no. 00882477). Cells were incubated in 10% CO_2 with 95% relative humidity (Appendix G). Cells were grown to about 75-100% confluency and transferred from 25 cm^2 growth area (T-25) to 75 cm^2 growth area (T-75) (Flask cat. #25115) and to 150 cm^2 (T-150) (corning, Tissue Culture Flask, polystyrene), respectively. When the cells in T-150 were 75-100% confluent, they were split to T-150 and fed with fresh growth medium. Four flasks of T-150, 75-100% confluent, were needed (enough for one fusion). DMEM with supplement containing 20% fetal calf serum (FCS)

(Hyclone Bovine Serum Lot #100355) was used to grow myeloma cells. The myeloma cell cultures were started 10 days before fusion. The day before fusion, the culture was fed with new medium to insure healthy cells. Growth of cells was evident when the media changed from pink to yellow, indicating acid production, or cells were checked microscopically for cell growth. To obtain myeloma cells for fusion, the flasks were shaken vigorously to dislodge cells from the bottom. The cells were collected by centrifugation at 800 rpm for 10 minutes. The supernatants were removed and sterilized by filtration for use as conditioned medium. The cell pellet was resuspended and washed in 50 ml DMEM with antibiotics by centrifugation at 800 rpm for 10 minutes. The cell pellet was resuspended with DMEM and the suspension was adjusted to contain 2×10^7 cells/ml. Cell viability was 97%. Cells were incubated in a 37 C water bath until fusion took place.

Hybridization

After the spleen cells and myeloma cells were prepared, fusion was accomplished as follows:

Polyetheylene glycol 1000 (8U218, J.T. Baker, TM grade, Lot #049385) was warmed to 37 C before use; DMEM was kept cold. The myeloma cells and spleen cells (1×10^8 cells each) were mixed in a 50 ml conical centrifuge tube (Corning, 50 ml/centrifuge tube with a screw cap, polypropylene 25330) and pelleted by centrifugation at 800 rpm for 10 minutes at room temperature. The supernatant was carefully aspirated from cells. The pellet was left as dry as possible. The tube was flicked to loosen the pellet and placed in a 37 C water bath. Fusing agent (0.2 ml

of 30% PEG 1000 in DMEM with 0.15 M HEPES) was added to the pellet and suspended by swirling. The suspended cells were centrifuged immediately at 800 rpm for 3 minutes at room temperature. The cell pellet was left exposed to PEG for 8 minutes. Five ml of DMEM with 0.15 M HEPES was added slowly with little suspension of the cell pellet. After 1-2 minutes, the cell pellet was suspended by intermittant swirling for 3-4 minutes, centrifuged at 800 rpm for 5 minutes, and the supernatant was aspirated. The cloning medium (Appendix D) (25 ml) was added to the fusion pellet. The cell pellet was left undisturbed for 6 minutes and then, gently suspended by swirling or with a pipette one time (no attempt was made to break all the cell clumps). Unfused spleen cells (2.5×10^7 cells) were added to the suspension and the volume was brought to 50 ml with cloning medium (Appendix D). The suspension was plated at 0.05 ml/well (1 drop with a 10 ml pipette) into ten 96-well tissue culture clusters seeded with 1.5×10^4 peritoneal macrophage cells/well. The plated cells were incubated undisturbed in a 10% carbon dioxide incubator for 4 days. At day 5 and day 12, 2 drops of medium were added to each well of the cloning plate with a 10 ml pipette. The clones appeared at approximately day 10. The well containing a clone was marked by looking in a mirror (Microtiter, Cook Engineering Company). Before collecting supernatant for testing, the cloning plates were checked in detail with a microscope for tiny clones which may have appeared late and could not be seen in the mirror.

Initial Screening for Positive Hybrids

Between 14-21 days after cell fusion, the supernatant media of cultures were harvested using a separate pipette for each well. Pipettes

were flamed before using. The supernatant solutions were tested undiluted for the presence of antibody of interest using the Indirect Immunofluorescence Antibody Test (IFA) as follows:

Indirect Immunofluorescence Antibody Test (IFA)

Preparation of Antigen. A 25 x 75 mm acetone resistant glass slide with 30 staggered wells 2 mm in diameter (Cell-Line Associates, Inc., Menisota, N.J.) was used for the IFA test. Formalin-treated cells for immunization were washed two times in 0.5% formalin in PBS as in the preparation of antigen above. Two microliters of diluted antigen suspension was dropped over the ringed areas of a FA slide. The smears were air dried and gently heat fixed.

Preparation of Antibody. A 1:10 dilution of serum of immunized mice was prepared in 0.2 M phosphate buffered saline, pH 7.6 (Appendix F). Doubling dilutions were made through 1:2048 in plain PBS. Rigid polystyrene U-bottomed microtitration plates (Linbro Scientific Co.) and an automatic diluter (Cooke Engineering Co.) were used. With a capillary tube, a drop of each serum dilution, or supernatants from tissue culture, was smeared in each well of the fixed antigen on the slide. The slide was incubated in a moist chamber (petri dish containing moist filter paper) at 37 C for 30 minutes. The slide was rinsed quickly with PBS and placed in a PBS bath for 15 minutes. It was removed from the bath, rinsed with distilled water for 7 minutes and gently blotted dry. A drop from a capillary pipette (approximately 20 microliters) of fluorescein isothiocyanate (FITC)-labeled goat antimouse immunoglobulin was placed in each well. The slide was incubated as above for 30 minutes. The slide

was rinsed quickly with PBS, placed in a PBS bath for 15 minutes, rinsed quickly with water, and gently blot dried. The antigen-antibody complex on the slide was fixed one minute with Kirkpatrick's fixative agent (Appendix F) and dried. Three drops of buffered glycerol (Appendix F) were added onto each FA slide and the slide was covered with a 24 x 60 mm cover glass (Corning). Slides were observed under oil immersion with a Leitz Dialux 20 fluorescence microscope equipped with an HBO-100 mercury incident light source and the Leitz I-cube filter system (2 x KP490 and a 1 mm GG 455 primary filter, TK 510 dichroic beam-splitting mirror and secondary filter).

Fluorescence intensities were read as follows:

4+ = Maximum fluorescence, brilliant yellow-green staining of bacteria cells.

3+ = Less bright yellow, green staining.

2+ = Less brilliant but definitive fluorescence.

1+ = Barely visible staining

$\pm$ = Questionable staining.

0 = No staining.

Tissue culture fluid was used undiluted in the IFA test for monoclonal antibody. Fluorescence above 1+ intensity indicated a positive clone for screening of positive hybrids. The serum titration end point was the highest dilution of serum that gave 1+ fluorescence intensity. The titer is the reciprocal of the dilution factor.

Direct Fluorescent Antibody Procedure (DFA)

This procedure was used to confirm antigen before immunization into mice and classification of 36 antigens for characterization of monoclonal antibody. The antigen for the DFA test was prepared as described in the IFA test. Twenty microliters of diluted antigen suspension was dropped over the ringed areas of a FA slide (Fluoro-Slide, A.S. Aloe Co.); smears were air dried and gently heat-fixed. Antigen smears were stained by the direct FA procedure. One drop of conjugate was spread evenly over the antigen, and the slides were kept in a moist chamber at room temperature for 30 minutes. They were rinsed with PBS and put in PBS for 15 minutes. They were then rinsed one minute in distilled water and air dried. Three drops of mounting fluid (Appendix F) were placed on the smear and a 24 x 60 mm cover slip applied. The slide was read as described above.

Cloning by Limiting Dilution

The most important principle for the recovery of stable active clones was to clone early and reclone frequently. Between 10-15 days after fusion, macroscopically visible colonies of hybrid cells appeared. Before cloning, the diameter of clones was approximately 2 mm. The clones were kept healthy by feeding with one-half volume of new medium the day before cloning. Only single clones with the highest fluorescent intensity (2+ - 4+) were selected. Feeder cells (peritoneal macrophages 1-4 x 10^4 cells/well were prepared on the day before fusion and checked for contamination before cloning. The selected clone was picked up in a sterilized pasteur pipette, dispersed, counted and serially diluted until the suspension contained an average of 5 cells/ml. One-tenth ml of this

dilution was added to each well to give an average of 0.5 cell/well.

Secondary and Tertiary Screening for Positive Hybrids

The methods and procedure were the same as for initial screening. The exception to this is that after the cells in 24-well plates had grown approximately to 75-100% confluency, the fluid was collected for the IFA test and the cells were transferred to T-25 flasks in DMEM medium with HAT. After the cells reached approximately 75-100% confluency in the T-25 flask, the fluid was tested again and the cells were transferred to a T-75 flask. The old flask was fed and continued to grow and the cells were frozen. The cells in the T-75 flask were fed with DMEM with hypoxanthine and thymidine, but without aminopterin. Tissue culture cells were grown to 75-100% confluency in the T-75 flask. The culture fluid was collected for monoclonal antibody characterization and the hybridoma cells were frozen as described below.

Freezing of Hybridoma Cells

To preserve hybridomas which produced an antibody of interest, cells were frozen at -80 C. The freezing medium contained 8% of dimethylsulfoxide (DMSO, Merk, West Germany). The hybridoma cultures were collected and centrifuged at 1000 rpm for 10 minutes. The cells were resuspended in cold freezing medium at approximately 10^7 cells/ml and placed into sterile 2 ml screw-cap vials. Vials were closed air-tight and placed in a styrofoam bag which had about 1 cm thick walls. Vials were put in -70 C for one day and transferred to a freezing box at a temperature below -80 C.

Freezing of Cloning Plates

After initial, secondary and tertiary screening for antibody and cloning by limiting dilution, the plates were fed one day before freezing by removal of one-half of the old medium from each well and adding two drops of fresh medium to each well. On the day of freezing, the medium was aspirated from each well and 0.05 ml of freezing medium was added. The plates were frozen as rapidly as possible at -80 C.

Characterization of Monoclonal Antibody

The monoclonal antibodies were characterized by their reactions against thirty-six antigens which were prepared in the following manner. Buffered charcoal yeast extract slants were innoculated with the appropriate L. pneumophila strains and incubated for 16 hours at 37 C. Cells were harvested in 1.0% formalin PBS solution. The suspensions were then incubated for 16-24 hours at 4 C to kill the cells. Killed cells were washed two times and resuspended in 0.5% formalin PBS to a concentration which yielded approximately 200 organisms per microscopic field smear under the 100 X objective. Strains of L. pneumophila were representative of serogroups 3 and 6 (Appendix B). Each antigen suspension was checked by DFA using absorbed conjugates to insure that the strains were assigned to the correct serogroup. Monoclonal antibodies from each clone were tested by IFA against each of the thirty-six antigens.

CHAPTER IV

EXPERIMENTAL RESULTS

Antigen Preparation

Failure of the antigen to grow on tryptic soy agar indicated that the L. pneumophila strain used for immunization was not contaminated by other organisms. Antigens used in IFA and DFA tests were tested with serogroup 6 and serogroup 3 - specific conjugates to ensure the organisms were categorized in the proper serogroup (Tables 1 and 2). Flagella were not observed in DFA or IFA tests. All antigen preparations were stained as expected. Cells of Chicago 2 varied in size.

Production of Antisera in Mice

Sera obtained from immunized mice via the supra orbital plexus were tested by IFA to determine the extent of the immune response. The titers of the antisera were not high (Table 3). Excluding mice 4, 6, 7, and 9, antibody titers did not increase following the 2nd immunization. Antibody titers of mice 2 and 3 decreased, and the antibody titer of mouse 1 did not change. A four fold increase in titer was observed in mouse 4. Even though the mice apparently did not respond well to the 2nd immunization, spleens of mice 1, 3, and 4 were selected as a source of lymphoid cells for use in fusion experiments $1ALDCH_2$ and $1BLDCH_2$.

For fusion experiment $2LDCH_2$, spleens of three mice (#5, 6 and 9) (Table 3) were selected as the source of immune cells. Generally, these mice responded better than the mice used in the other fusions. The end point titers were higher and the anamnestic response more pronounced.

Table 1. Categorization of antigens in serogroup 6 by DFA test[a]

Antigens	IFA values against Bloomington 2 serogroup 3-specific conjugate	IFA values against Chicago 2 serogroup 6-specific conjugate
UCH 5	0	3+
SF 7	0	3+
80-011839	0	3+
Chicago 3	0	4+
Toronto 794	0	3+
Indy 4	0	3+
UCH 14	0	3+
GU 11560 1A	0	3+
SF 5	0	3+
UCH 10	0	4+
Adelaide 3	0	4+
Oxford 2 10-17	0	4+
UCH 2	0	4+
Denver 2	0	4+
UCH 11	0	4+
UCH 7	0	4+

Table 1. (continued)

Antigens	IFA values against Bloomington 2 serogroup 3-specific conjugate	IFA values against Chicago 2 serogroup 6-specific conjugate
Cleveland 2	0	4+
Houston 2	0	4+
UCH 8	0	4+
Concord 2	0	2+[b]
UCH 13	0	4+
UCH 4	0	4+
SF 4	0	3+
Pittsburgh 8	0	3+
UCH 6	0	4+
Greenfield 1	0	3+
Oxford 1	0	4+
UCH 3	0	4+
Chicago 4	0	2+[c]
Albany 1	0	2+[d]

[a] These antigens were tested against serogroup specific conjugate by DFA at least 4 times.
[b] Concord 2 strain shows less fluorescent intensity.
[c] Chicago 4 strain shows less fluorescent intensity.
[d] Albany 1 strain shows less fluorescent intensity.

Table 2. Categorization of antigens in serogroup 3 by DFA test[a]

Antigens	IFA values against Bloomington 2 serogroup 3-specific conjugate	IFA values against Chicago 2 serogroup 6-specific conjugate
UCH 9	4+	0
Detroit 5	4+	0
SRP 8	4+	0
Burlington 4	3+	0
UCH 1	4+	0
UCH 12	4+	0

[a] These antigens were tested against serogroup specific conjugate by DFA at least 4 times.

Table 3. Titer of mice after injection with single and double dose with Chicago 2 and Bloomington 2 strain

Mouse number	Dose	D.F. 1:10	1:20	1:40	1:80	1:160	1:320	1:140	1:1280	1:2560	IFA values against Bloomington 2 strain
		IFA values against Chicago 2									
1*	S.D.[a]	3+	3+	3+	2+	1+	±	0	0	0	0
	D.D.[b]	3+	3+	2+	1+	1+	±	0	0	0	0
2	S.D.	4+	4+	3+	2+	2+	1	±	0	0	0
	D.D.	3+	3+	3+	2+	1+	0	0	0	0	0
3*	S.D.	4+	4+	3+	2+	1+	±	0	0	0	0
	D.D.	4+	4+	3+	1+	0	0	0	0	0	0
4*	S.D.	2+	±	0	0	0	0	0	0	0	0
	D.D.	2+	2+	1+	0	0	0	0	0	0	0
5*	S.D.	NT[c]	NT	NT	NT	NT	NT	NT	NT	NT	0
	D.D.	3+	3+	3+	3+	3+	3+	0	0	0	0
6*	S.D.	3+	2+	1+	±	0	0	0	0	0	0
	D.D.	3+	3+	2+	2+	1+	0	0	0	0	0

Table 3. (continued)

Mouse number	Dose	D.F. 1:10 IFA values against Chicago 2	1:20	1:40	1:80	1:160	1:320	1:140	1:1280	1:2560	IFA values against Bloomington 2 strain
7	S.D.	3+	3+	3+	3+	3+	2+	1+	±	0	0
	D.D.	3+	3+	3+	3+	3+	2+	2+	1+	0	0
8	S.D.	3+	3+	3+	2+	2+	1+	0	0	0	0
	D.D.	3+	3+	2+	2+	2+	0	0	0	0	0
9*	S.D.	2+	2+	2+	2+	2+	±	0	0	0	0
	D.D.	3+	3+	3+	3+	3+	2+	2+	1+	0	0

[a]S.D. is the single dose (Immunization I/P with complete Freunds).
[b]D.D. is the double dose (Boost I/V)
[c]NT is not tested due to the failure to collect blood
*These mice were selected to obtain spleen cells.

The secondary responses in mice 6 and 9 were 4 and 8 times the primary response, respectively.

Initial screening for positive hybrids. Results of the initial screening for positive clones by IFA are shown in Figure 2. The total number of clones appearing was 145. Five of them did not produce antibody against either Chicago 2 or Bloomington 2. Sixty-four clones gave 1+, 59 clones gave 2+, 15 clones gave 3+, and 6 clones gave 4+ fluorescent intensity against Chicago 2 antigen. All the of supernatants tested were negative to Bloomington 2 antigen (data not shown).

Twenty-five clones were selected on the basis of their positive IFA reaction and recloned by limiting dilution (Table 4). The first clones appeared approximately ten days after plating and they grew for three weeks before testing for antibody production. Only nine of the original positive hybridomas continued to produce antibody after the cloning process (Table 4).

Secondary screening for positive hybrids. Results of IFA testing are shown in Figure 3. The total number of clones appearing were 550. Fifty percent of clones did not produce antibody. Ten of the positive clones were recloned a second time.

Tertiary screening for positive hybrids. Fifty to eighty percent of the fluids from cells with clones gave 4+ fluorescent intensity (Figure 4). The total number of clones which appeared in each plate varied from plate to plate (Table 5). Most of the clones appeared within 2 weeks of incubation. Almost 100% of the clones from the tertiary screening produced monoclonal antibodies against Chicago 2 strains.

Fig. 2. Reactivity of clones against Chicago 2 obtained from initial screening of fusions $1ALDCH_2$, $1BLDCH_2$, and $2LDCH_2$

0 = negative
1+ = very weak positive
2+ = weak positive
3+ - 4+ = definite positive

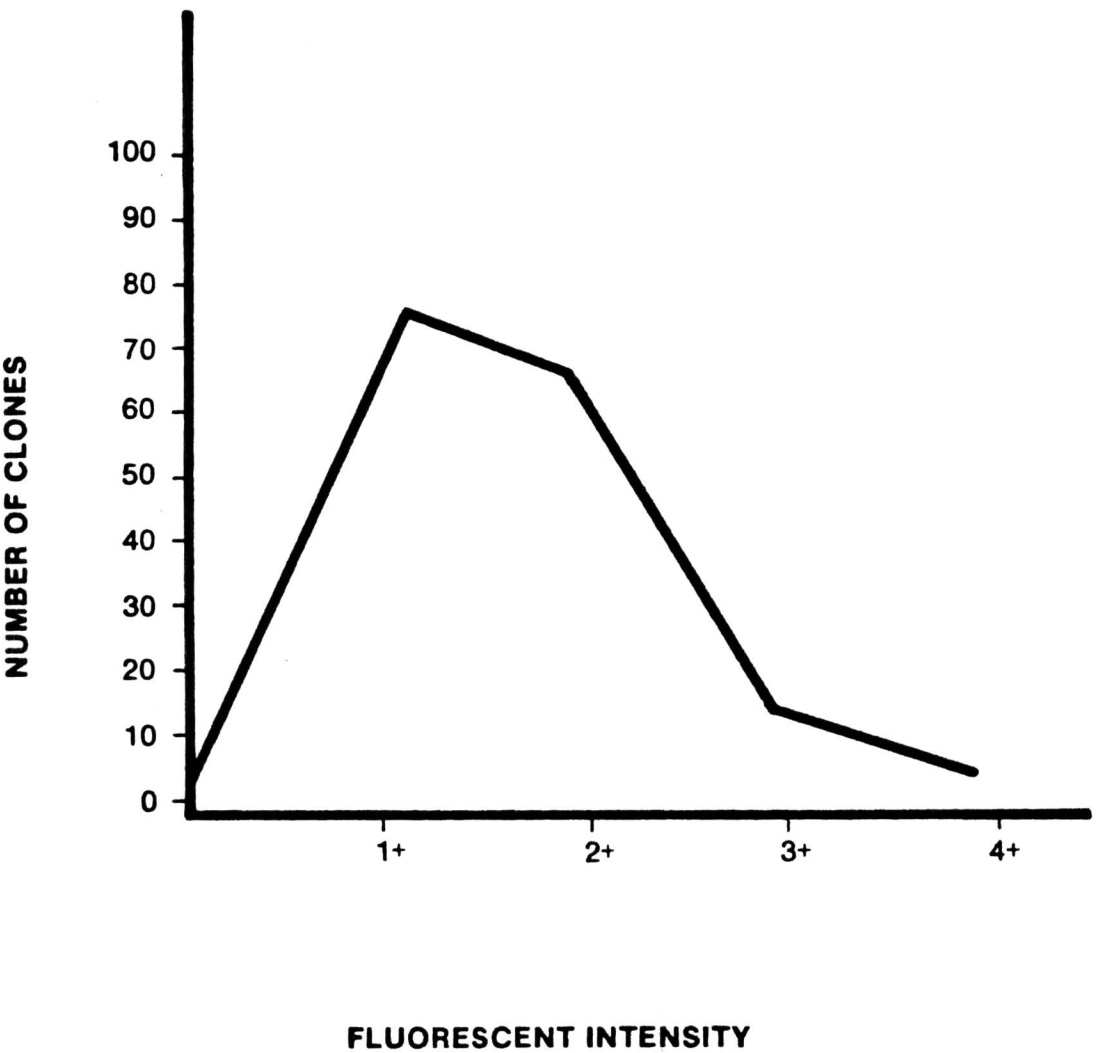
NUMBER OF CLONES
100
90
80
70
60
50
40
30
20
10
0
1+
2+
3+
4+
FLUORESCENT INTENSITY

Table 4. Record of first recloning and IFA results of secondary screening

Clone		Plated (cells/0.2 ml)	Total number of clones appearing	Total number of positive clones
1ALDCH$_2$[a]	1A12	0.5	6	0
	1B12	1.0	4	0
	1D9	0.98	22	0
	1G7	1.0	12	0
	2H4	0.9	8	0
	2H11	0.1	30	0
	3B2*	1.0	29	29
	3E11*	1.0	31	28
	4B1	1.03	26	0
	5A5	1.0	14	0
1BLDCH$_2$[b]	3A11	0.5	28	0
	3C3	0.5	0	0
	3C6*	1.0	26	20
	4F1	0.42	0	0
	4F10	1.0	9	0
	4F11	0.74	15	0
	4F12	0.5	53	0

Table 4. (continued)

Clone		Plated (cells/0.2 ml)	Total number of clones appearing	Total number of positive clones
	5A1	1.0	22	0
	5C1	1.0	4	0
$2LDCH_2$[c]	1H7	0.5	39	39
	1H11*	0.5	60	60
	2H5	0.5	13	13
	4D1*	1.0	65	65
	4E4	0.5	6	6
	5C1*	1.0	30	27

[a] $1ALDCH_2$ is the experiment number 1 part A, fusing agent containing 0.15M HEPES.
[b] $1BLDCH_2$ is the experiment number 1 part B, fusing agent containing no HEPES.
[c] $2LDCH_2$ is the experiment number 2, fusing agent containing 0.15M HEPES.
*These clones were selected to reclone.

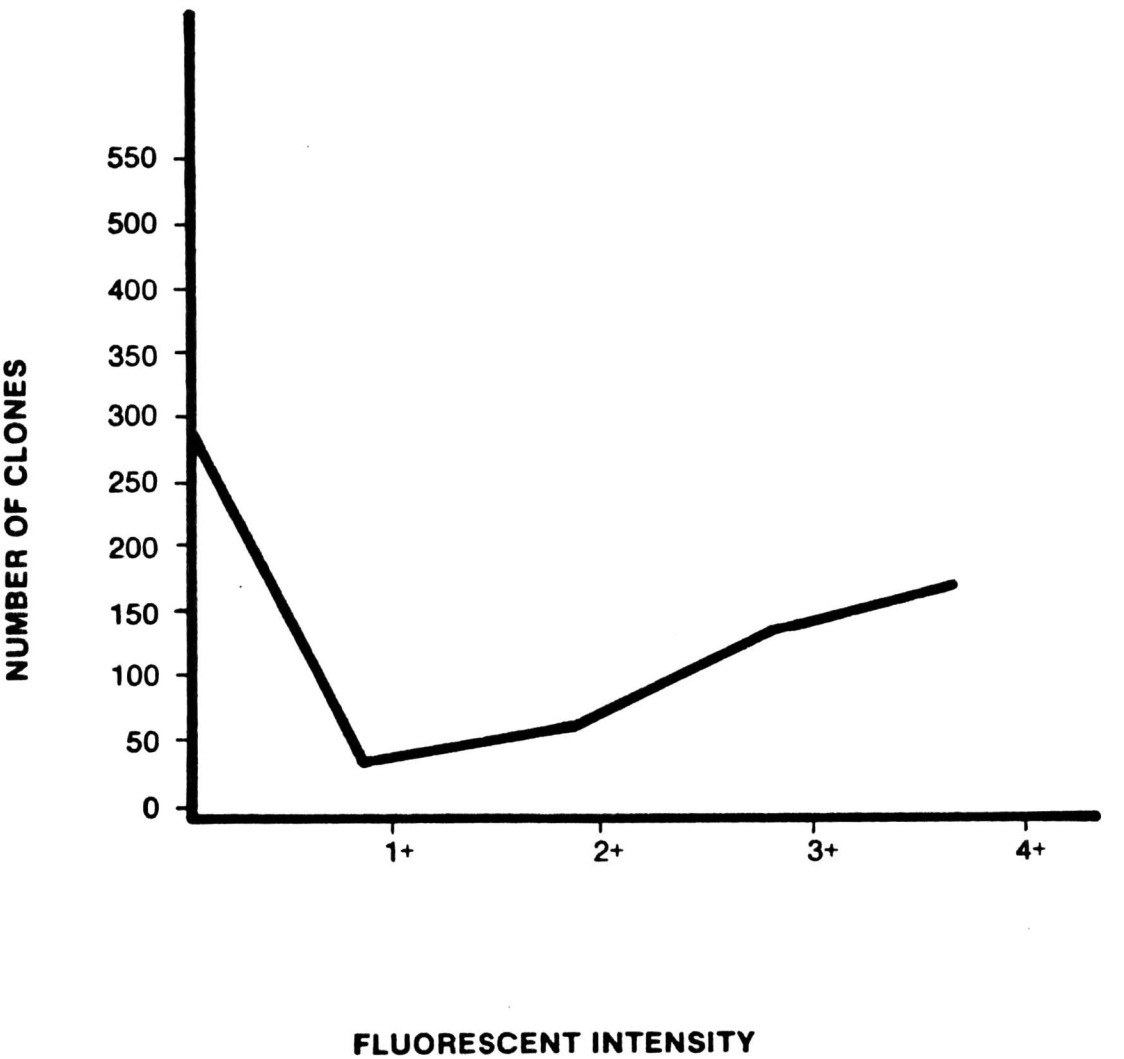
NUMBER OF CLONES
550
500
400
350
300
250
200
150
100
50
0
1+
2+
3+
4+
FLUORESCENT INTENSITY

Fig. 4. Reactivity of clones against Chicago 2 obtained from tertiary screening of fusions $1ALDCH_2$, $1BLDCH_2$, and $2LDCH_2$

0 = negative
1+ = very weak positive
2+ = weak positive
3+—4+ = definite positive

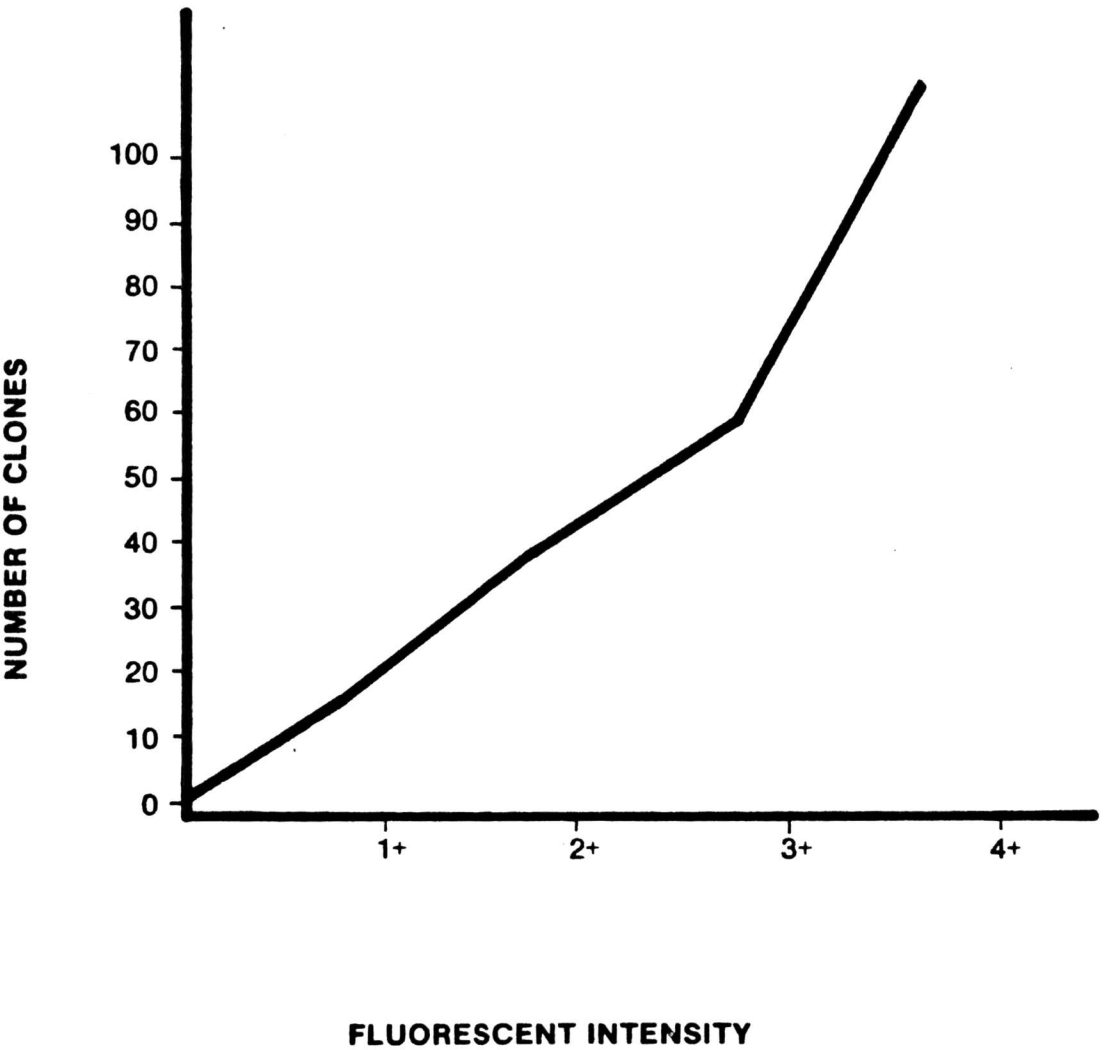
NUMBER OF CLONES
100
90
80
70
60
50
40
30
20
10
0
1+
2+
3+
4+
FLUORESCENT INTENSITY

Table 5. Record of second recloning and IFA results of tertiary screening

Clone		Plated (cells/0.2 ml)	Total number of clones appearing	Total number of positive clones
1ALDCH$_2$	3B2-2B10	0.5	54	54
	3E11-2E7	0.5	48	48
1BLDCH$_2$	3C6-1D7	0.5	24	22
	3C6-1H9	0.5	59	59
2LDCH$_2$	1H11-1C2	0.5	50	50
	1H11-1B3	0.5	45	45
	4D1-1C10	0.5	36	36
	4D1-1F2	0.5	55	55
	4D1-1F3	0.5	56	56
	5C1-1B8	0.5	4	4

The ten monoclonal hybridomas were then expanded after tertiary screening. The fluids were collected after 3 days of culture on confluent monolayers. The monoclonal antibodies were then tested against 30 strains of L. pneumophila serogroup 6, and 6 strains of L. pneumophila serogroup 3.

Characterization of Monoclonal Antibodies

Table 6 summarizes the data obtained from analysis of reactivity patterns of ten monoclonal antibodies. Seven antigens, Indy 4, UCH 10, UCH 2, UCH 4, UCH 8, UCH 13, and UCH 11 reacted very well with all of ten monoclonal antibodies. UCH 14, Cleveland 2, UCH 3, Oxford 1, and Houston 2 reacted strongly with monoclonal antibodies numbers 6-10. Concord 2, Chicago 4 and Albany 1 gave negative results to most of 10 monoclonal antibodies. All six strains of L. pneumophila serogroup 3 gave negative results to all monoclonal antibodies.

Table 6. Indirect Fluorescent Antibody Reactivity Patterns of Ten Monoclonal Antibodies with 30 strains of L. pneumophila serogroup 6

	Monoclonal Antibody[a]									
Representative Strain	1	2	3	4	5	6	7	8	9	10
Serogroup 6										
1. UCH 5	3[c]	3	3	3	3	3	3	3	3	3
2. SF 7	3	3	3	3	3	3	3	3	3	3
3. 80-011839	3	3	3	3	3	3	3	3	3	3
4. Toronto 794	3	3	3	3	3	2[d]	3	3	3	3
5. Indy 4	4[b]	4	4	4	4	4	4	4	4	4
6. UCH 14	3	3	3	3	3	4	4	4	4	4
7. GU 115601	3	3	3	3	3	3	3	3	2	3
8. SF 5	3	3	3	3	3	3	3	3	3	3
9. UCH 10	4	4	4	4	4	4	4	4	4	4
10. Oxford 2	3	3	3	3	3	3	3	3	3	3
11. Adelaide 3	3	3	3	3	3	3	3	3	3	3
12. UCH 2	4	4	4	4	4	4	4	4	4	4
13. Denver 2	3	3	3	3	3	3	3	3	3	3
14. UCH 7	3	3	3	3	3	3	3	4	3	3
15. Cleveland 2	3	3	3	3	3	3	3	4	4	4
16. Concord 2	4	-[e]	-	-	-	3	-	3	-	-
17. UCH 4	4	4	4	4	4	4	4	4	4	4

Table 6. (continued)

Representative Strain	Monoclonal Antibody[a]									
	1	2	3	4	5	6	7	8	9	10
Serogroup 6										
18. SF 4	3	3	3	3	3	3	3	3	3	4
19. Pittsburgh 8	3	2	3	3	3	3	3	3	3	3
20. UCH 6	3	3	3	3	3	3	3	3	3	3
21. Greenfield 1	4	3	3	3	3	3	3	3	3	3
22. Oxford 1	3	3	3	3	3	4	3	3	4	3
23. UCH 3	3	3	4	4	4	4	4	4	4	4
24. Chicago 4	3	2	-	-	-	-	-	2	-	-
25. UCH 8	4	4	4	4	4	4	4	4	4	4
26. UCH 13	4	4	4	4	4	4	4	4	4	4
27. Houston 2	3	3	3	3	3	4	4	4	4	3
28. UCH 11	4	4	4	4	4	4	4	4	4	4
29. Chicago 3	3	3	3	3	3	3	3	3	3	3
30. Albany 1	-	-	-	-	-	-	-	2	2	-

[a] Number designation given to the monoclonal antibodies which were produced by hybridomas 1BLDCH$_2$3C61H9, 1ALDCH$_2$3B2-2B10, 1ALDCH$_2$3E11-2E7, 1BLDCH$_2$3C6-1D7, 2LDCH$_2$4D1-1F3, 2LDCH$_2$4D1-1F2, 2LDCH$_2$1H11-1C2, 2LDCH$_2$4D1-1C10, 2LDCH$_2$5C1-1B8, and 2LDCH$_2$1H11-1B3, respectively.

[b] Bright fluorescence

[c] Definite positive

[d] Weakly positive reaction

[e] Negligible fluorescence.

CHAPTER V

DISCUSSION

The procedure proposed in this investigation offers an alternative to conventional serological techniques used for infectious disease in most laboratories. Monoclonal antibodies for detection of infectious disease is a relatively new immunologic technique. This research objective was to use hybridoma techniques derived by various investigators to allow production of monoclonal antibodies against cell surface antigens of L. pneumophila serogroup 6 (Chicago 2 strain), to determine whether there is antigenic heterogeneity among strains of L. pneumophila serogroup 6 (Chicago 2 strain), and to determine whether monoclonal antibodies to serogroup 6 might be useful as diagnostic reagents for detecting legionellosis. L. pneumophila serogroups 3 and 6 appear to be widely distributed geographically. These two serogroups share common antigens as indicated by strong cross reactivities when tested in the DFA test using conjugate against the heterogenous strain. The results (Table 6) show that ten monoclonal antibodies produced by hybridomas derived from the fusion of mouse myeloma cells and spleen cells of mice that have been immunized with Chicago 2 strain (serogroup 6) will react only with the antigens in serogroup 6. None of these ten monoclonal antibodies reacted with antigens in serogroup 3 (data not shown). Seven strains of L. pneumophila serogroup 6 reacted very well with all ten monoclonal antibodies. Two additional strains gave relatively high fluorescent intensity with monoclonal antibodies number 6-10.

Three strains (Concord 2, Chicago 4, and Albany 1) did not react with monoclonal antibodies designated, 3,4,5,6,7,9 and 10. Concord 2, Chicago 4, and Albany 1 produced weak reactions with absorbed polyclonal antibodies (Table 1).

With current technology, many investigators suggest that they would not attempt a fusion unless they have animals whose serum titers had risen about 100 fold, three days after boost. Results of these experiments indicate that monoclonal antibodies can be obtained with mice having lower titers and lower secondary responses. The group of mice (mice #1, 3 and 4) used for the first fusion (fusion # $1ALDCH_2$ and $1BLDCH_2$) had serum titers of 160, 80 and 40, respectively, and very little secondary response. Mice used for the second fusion ($2LDCH_2$) had serum titer increases of up to 8 fold, three days after boost. Results from Table 4 show that only 2 clones (3B2 and 3E11) from $1ALDCH_2$ and only one clone (3C6) from $1BLDCH_2$ continued to produce antibody, the remaining 16 clones failed to produce antibody. From the second fusion, $2LDCH_2$, every clone continued to produce antibody. Loss of secretors may be due to overgrowth by non-secretors or to loss of chromosomes. In cases where detectable levels of serum antibody are low it could be explained that in the second dose of immunogen all of the antigens have not cleared. Therefore, antigen-antibody complex may be in a form such that antibody cannot be detected. The titer may already be at a maximum.

It is of interest that in this experiment we did not detect any clone producing antibody that was reactive with L. pneumophila serogroup 3 strains, although cross reactivities between Chicago 2 and Bloomington 2

antigens have been observed with rabbit antibodies (McKinney, et al., 1979). From experimental results in Table 3 serum antibody from mice immunized with Chicago 2 did not react with Bloomington 2 antigen. It appears that the mice in this study did not produce antibody against the common antigenic determinants which are shared between these two serogroups.

It is possible that radioimmunoassay (RIA) may be superior to the FA method for screening hybridoma fluids although both methods are analogous in concept and sensitivity. Only hybridomas producing antibodies to cell surface antigens can be detected by the IFA method. If a RIA had been used to screen for antibody producing hybridomas, antibodies to both surface and internal antigens of L. pneumophila (Chicago 2) could have been detected. Furthermore, the evaluation of FA slides is laborious and time consuming and also depends on the judgment of the individual for accurate interpretation. When the eye of the inspector is fatigued, positive clones may be judged as negative and result in failure to detect clones that produce antibody which has cross reactivity with Bloomington 2 antigen.

Antigens used for detection of antibody in supernatants played an important role in detection of monoclonal antibody. After harvesting an antigen from the culture medium, this antigen must be washed to produce an antigen which will not bind non-specifically to the conjugate.

The amounts of antigens on the FA slide were found to be important. It is shown that there are limiting quantities of antigen which are appropriate on the FA slide and give optimum fluorescent intensity with antibody. Excess antigen on the FA slide can reduce the apparent

fluorescent intensity. It is my observation that approximately 200 cells per microscopic field (1,250X) gave the best result for characterizing monoclonal antibodies. When the optimum concentration of antigen is obtained, the specific antibody reaction is demonstrably greater.

The results (Fig. 2) provide evidence that production of antibody specific for the immunogen is relatively low after hybridization (initial screening). More than 50% of positive clones gave 1+ and 2+ fluorescent intensity against Chicago 2 antigen. Ten percent gave 3+ and only 4% gave 4+ fluorescent intensity. The purpose of cloning and recloning is to isolate the secretor cells from the non-secretors and to obtain a truly monoclonal cell line. The fluorescent intensity is usually higher when all cells are producing antibody. Also, the cloning procedure allows one to select out or remove cells that may have originally been secretors but can no longer secret due to loss of chromosomes.

Specificity should not change unless antibodies of two different specificities are being produced by different hybridomas. Also, the quality of the antibody that is produced does not change. The concentration of the antibody in the fluid will vary depending on the number of cells that are secreting. After the secondary screening (Fig.3) a number of clones gave 3+ and 4+ fluorescence intensity which was higher than in the initial screening, and in the tertiary cloning and screening (Fig.4), more clones produce 3+ and 4+ reacting antibodies. This is because the cells represent a true clone, and non-secretors have been removed. Also, the growth rate of the clones may increase as they adapt to culture. IFA test results show that after the tertiary cloning

almost 100% of the clones gave positive results. The percentage giving 4+ fluorescent intensity varied from 50-80%. In the cases where cloning plates failed to produce antibody, hybridomas from 24 well plates were used to reclone as soon as possible. After the selected clones were aspirated for recloning, every plate was frozen to preserve the remaining untested clones for future investigations.

In Table 4 it may be seen that more than 50% of the clones that initially secreted antibody became non-secretors after the first cloning. This instability is due in part to the loss of heavy chain and light chain synthesis associated with chromosome loss and/or overgrowth by non-secreting clones or variants. The rate of loss of production of antibody in the experiment was high and the similar to that observed by other investigators (Kwan, et al., 1980; Goding, 1980).

There are a lot of problems facing investigators in hybridoma technology. Observations from this study indicate that some problems are not yet solved. Some factors are still not clear. The exact role of macrophages as feeder cells is still not completely understood. Every step must be kept in mind. That is very important. Before starting to work each step must be understood. The screening method must be decided before the experiment is begun and all the materials should be available promptly. When contamination such as mold growth is observed in any well the entire plate should be discarded. It is not advisable to save that plate because it may be spread throughout the whole experiment. The contaminating problem can be reduced by using aseptic techniques.

CHAPTER VI

SUMMARY

1. Mice that give a strong secondary immune response to the immunogen are better for fusion experiments than mice that give a weak secondary response.
2. Maximum fluorescece was obtained in IFA test with culture fluids of hybridomas after the second recloning. Further cloning did not result in any increase in fluorescence intensity in the IFA test with culture fluids, suggesting that the monoclonal cell lines of hybridomas were obtained after two reclonings.
3. All ten monoclonal antibodies react specifically to strains of L. pneumophila serogroup 6 but not to strains of serogroup 3. This provides evidence that monoclonal antibodies can solve cross-reactivities problems between these two serogroups.
4. Monoclonal antibodies provide a means for examining the antigenic variation among strains of bacteria in the same serogroup.

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APPENDIX A

Myelomas Used for Hybridoma Production

Name	Ig Synthesized or Cell Type
P3 x 63 Ag 8	$I_gG_1(K)$
P3-NS1/1 Ag 4-(NS1)	(-)(K)
MPC 11 - X45 - 6TG	$I_gG2b(K)$
Sp2/0-Ag 14	None
Sp1	I_gM
Sp2Ag14	I_gG2b
Sp6	I_gM
Sp7	$I_gM(K)$
Sp25 - 4 Ag	$I_gG(K)$
45.6 TG 1.7	I_gG2b
4TO61L1	K
X63 Ag 8.635	None
FO	None
210.RCY 3-Ag7	K
BW 5147	Thymic lymphosarcoma
E1-4	Thymic lymphosarcoma

APPENDIX B

Antigens Used for Characterization

1. Antigens in serogroup 6

   UCH 5

   SF 7

   80-011839

   Toronto 794

   Indy 4

   UCH 14

   GU 115601A

   SF 5

   UCH 10

   Oxford 2 10-17

   Adelaide 3

   UCH 2

   Denver

   UCH 7

   Cleveland 2

   Concord 2

   UCH 4

   SF 4

   Pittsburg 8

   UCH 6

Greenfield 1

Oxford 7

UCH 3

Chicago 4

UCH 8

UCH 3

Houston 2

UCH 11

Chicago 3

Albany 1

2. Antigens in serogroup 3

UCH 12

Burlington 4

UCH 9

Detroit 5

UCH 1

SRP 8

APPENDIX C

Preparation of HAT

1. Stock solution (100X) of Hypoxanthine and Thymidine (HT) pH 9-9.5

   a. Hypoxanthine (H) (6-hydroxypurin Sigma grade)
      M.W. 136.1 Final concentration 10^{-4}M — 136.1 mg

   b. Thymidine (T) (Sigma grade, crystalline)
      M.W. 242.2 Final concentration 3×10^{-5}M — 76 mg

   c. Mix and bring volume up to 100 ml with cell culture water (double distilled water)

   d. Filter through 0.2 micron filter pore (Sybron/Nalge)

   e. Freeze at - 80C.

2. Stock solution (100X) of (+) Aminoptherin (A)

   a. Aminopterin (A) (Sigma grade Lot #32F 0485 anhydrous form 4-amino-10-methyl folic acid;4-amino-N^{10}-pteroyglutamic acid) pH 7.8, Final concentration 10^{-5} M — 100 mg

   b. Bring volume up to 200 ml with double distilled water

   c. Filter and freeze at -80C

APPENDIX D

Preparation of Growth Medium

1. Supplemented Dulbecco's Modified Eagle Medium (DMEM)

a.	DMEM	400 ml
b.	Fetal Bovine Serum (FBS)	100 ml
c.	Antibiotics (Penicillin 10,000 unit/ml, Streptomycin 10,000 unit/ml)	5 ml
d.	Glutamine (200 mM)	5 ml
e.	2 Mercaptoethanol (0.1M)	2.5 ml
f.	Sodium Pyruvate (100 mM)	5 ml
g.	Mix well in 500 ml Wheaton Screw Cap bottle	
h.	Filter with Nalgene Filter Unit Type LS 0.2 Micron (Sybron/Nalge)	
i.	Store at 4 C no longer than two weeks	

2. Cloning Medium

a.	DMEM	250 ml
b.	HT(100X) (Appendix C)	5 ml
c.	A(100X) (Appendix C)	5 ml
d.	Conditioned Medium (This medium collected from growing myeloma cell)	250 ml
e.	Mix and filter	
f.	Store at 4 C	

3. Preparation of Growth Medium Containing HAT

a.	Growth Medium	500 ml
b.	HT (100X) (Appendix C)	5 ml
c.	A (100X) (Appendix C)	5 ml
d.	Mix and filter	
e.	Store at 4 C	

APPENDIX E

Preparation of Polyethylene Glycol

1. Use 8U218 J.T. Baker TM Grade (MW-1000). Lot #049385,
2. Melt and sterilize by autoclaving for 30 minutes.
3. Mix cool but liquified PEG, 3 ml

   DMEM 5.95 ml

   1M HEPES (N-2-Hydroxyethyl piperazine-N-2-ethan-sulfonic acid.) 1.05 ml.
4. Adjust pH to 8.2
5. Store frozen.
6. Warm up at 37 C in water bath before adding into fusion pellet.

APPENDIX F

Immunofluorescence Buffers, Fixative Agent and Mounting Fluids

1. Phosphate Buffer Saline (PBS), pH 7.6, 0.01M:

   a. Concentrated Stock Solution (pH 7.6)

$NaH_2PO4\ H_2O$	7.20 gm
Na2HPO4 (anhydrous, reagent grade)	49.44 gm
NaCl (reagent grade)	340.00 gm
Distilled water to make final volume	4 liter

   b. Working Solution (pH7.6, 0.01M)

Concentrated stock solution	100 ml
Distilled water to make final volume	1 liter

   c. 1% formalin PBS

PBS working solution	99 ml
Formalin (37%-38%)	1 ml

   d. 0.5% formalin PBS

PBS working solution	99.5 ml
Formalin (37%-38%)	0.5 ml

2. Glycerol mounting fluid: Buffered glycerine (pH 8.3)

(FA mounting medium)

0.2M Na_2HPO4	10 ml
Glycerine (reagent grade)	90 ml

Combine and mix by stirring (do not shake)

3. Kirpatrick's fixative agent

Absolute ethyl alcohol	60 ml
Chloroform	30 ml
Formalin (37%)	10 ml

APPENDIX G

Carbon Dioxide Incubator

10% carbon dioxide

95% relative humidity (distilled water supply containing 10 mg/l cycloheximide)

37 C temperature